Treatment with a nucleoside polymerase inhibitor reduces shedding of murine norovirus in stool to undetectable levels without emergence of drug-resistant variants

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Running title: 2CMC reduces shedding of MNV without drug resistance

Abstract

Prolonged norovirus shedding may occur in certain patients, such as organ transplant recipients. We established a mouse model for persistent norovirus infection [using mouse norovirus (MNV/CR6 strain). The nucleoside viral polymerase inhibitor 2'-*C*-methylcytidine (2CMC) [but not by favipiravir (T-705)] reduced viral shedding to undetectable levels. Viral rebound was observed after stop of treatment, which could again be effectively controlled by treatment with 2CMC. No drug-resistant variants emerged.

Noroviruses are an important cause of chronic gastroenteritis in immunocompromised patients (1). Although most norovirus infections are acute and self-limiting, the infection can become chronic in (i) solid organ and hematopoietic stem cell transplant recipients, (2-4), (ii) patients undergoing chemotherapy (3, 5, 6) and with immunodeficiencies, including those caused by HIV infection (7-10). Prolonged norovirus infection can also be observed in young children (11) and elderly (12, 13). Reduction of immunosuppressive therapy is, when feasible, the strategy of choice to control the infection in transplant recipients. Specific antiviral therapy to treat (chronic) norovirus gastroenteritis is not available. The effect of drugs such as ribavirin and nitazoxanide has been evaluated in a small number of patients but no clear beneficial clinical effect was observed (10, 14, 15). We wanted to establish persistent mouse norovirus (MNV) infection model that can be used to assess the efficacy of norovirus inhibitors on such infection. To that end the MNV.CR6 (herein CR6) strain was employed. The MNV is a genogroup V norovirus that has been widely used as a surrogate for human noroviruses (16, 17); it comprises about 30 strains of which the CR6 is avirulent but replicates for weeks to months in wild-type and (to higher titres) in innate immune deficient- mice (18, 19). Interferon receptor knockout mice (AG129) (20) were infected orally with the CR6 strain and the effect of two small molecule inhibitors of *in vitro* norovirus replication [2'-*C*-methylcytidine (2CMC) and favipiravir (T-705)] on viral shedding in stool was assessed (21, 22). We demonstrated earlier that 2CMC is effective in the treatment and prophylaxis of acute MNV-infection in AG129 mice (23).

2CMC was synthesized as described (24) and was dissolved in sterile saline. T-705 was purchased from BOC Sciences (New York, USA) and was formulated in 0.4%

carboxymethylcellulose (Sigma–Aldrich, Bornem, Belgium). The MNV, strain MNV.CR6 (kindly provided by Dr. Virgin, Washington University, St. Louis, USA) was propagated in RAW 264.7 as described before (22).

AG129 mice (129/Sv mice) originally from BK Universal, UK, were bred and housed at the Rega Institute under specific-pathogen-free conditions. All experiments were performed under the guidelines and authorization of the Ethical Committee of the University of Leuven (P101/2012). For all experiments, age- and sex-matched mice, 8–12 weeks of age were infected by oral gavage with 10^6 $CCID_{50}$ (50% cell culture infectious dose) of CR6. At 7 days post-infection (pi) mice were either left untreated (n=9) or treated with 100 mg/kg/day of 2CMC by the subcutaneous route for 5 (n=4), 7 (n=4) or 11 (n=4) days. Next, two more rounds of 14-day treatments (with a ~4-week interval in between) with 2CMC (n=10) or with T-705 [200 mg/kg/day by oral gavage] (n=5) were given. At each day after infection, the general condition and weight of treated and untreated mice was assessed and stool was collected individually (whenever possible during one daily period of observation), scored for consistency (0, normal feces; 1, mixed stool samples containing both solid and pasty feces; 2, pasty feces; 3, semiliquid feces; 4, liquid feces) and levels of MNV RNA were quantified by qRT-PCR. RNA was extracted from stool samples, using the RNeasy minikit (Qiagen, The Netherlands) according to the manufacturer's protocol. For the MNV qRT-PCR, primers and probe were used as described before with minor modifications. (22) The iTaq Universal Probes reaction mix (Bio-Rad, Belgium) was used and cycling conditions were: reverse transcription at 50 °C for 10 min, initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 30 s (Roche LightCycler 96, Roche

Diagnostics, Belgium). Statistical analysis was performed using Prism 5 software (Graph-Pad Software, San Diego, CA). [***p <0.001, ** p <0.01, * p<0.05, and ns no statistically significant difference (p≥0.05)]. p-values were determined with the nonparametric Mann-Whitney test.

Following oral infection with CR6, all AG129 mice remained healthy and did not show symptoms that are typically observed in mice infected with MNV-1, a strain that causes acute infections (e.g. ruffled fur, reduced activity, squinted eyes, diarrhea) or weight loss (Fig. 1A). The consistency of the stool of infected animals remained normal (score 0-1) throughout the experiment (data not shown) but viral RNA was detected from day 2-3 pi onwards in some of the animals (Fig. 1B). From day 6 pi on, viral RNA was consistently detected in the stool of all infected mice at high titres (in most mice > 4 $\log_{10}$ RNA copies /g of stool). Hence, day 7 pi was selected as the start of treatment with 2CMC for periods of respectively 5, 7 or 11 consecutive days (Fig. 2A). Shedding of virus in the stool was quantified to monitor whether the inhibitor(s) can (i) reduce or even stop viral shedding and (ii) completely cure the animal from the infection. Treatment with 2CMC (Fig. 2) resulted in a significant reduction of virus shedding in the stool as soon as one day after the start of treatment (day 8 pi) [reduction of 1.7 $\log_{10}$ RNA copies /g of stool in 2CMC-treated mice versus untreated mice]. At day 14 pi, i.e. after 7 days of treatment, CR6 RNA had become undetectable in the stool of all the 2CMC-treated mice. Viral RNA remained undetectable in the stool of the 2CMC-treated animals until the end of treatment at day 17 pi [end of the 11-day treatment] (Fig. 2A). However, 3 days after stop of the 11-day 2CMC-treatment period, viral RNA became again detectable in the stool of mice, at levels comparable to those of the untreated control (Fig 2D). Hence, a 2CMC-treatment for ≤ 11

days proved not sufficient to completely eliminate CR6 from the infected mice. Also soon after cessation of the 5 or 7-day treatment, “rebound” was observed in all mice.

Next, the effect of a treatment scheme consisting of two rounds of 14 consecutive days of 2CMC-treatment (with a pause of 4 weeks in between) was evaluated; in parallel the effect of favipiravir [of which we demonstrated earlier *in vitro* anti-MNV activity, (22)] was assessed (Fig. 3). In 6 of 10 mice, CR6 RNA was undetectable in stool of 2CMC-treated mice following another 14 days of treatment (day 21 pi). Akin to the experiment presented in Fig. 2, a rebound in viral RNA was noted following stop of treatment. Favipiravir did not reduce viral shedding (Fig. 3A). The dose selected for the study was determined on the basis of the solubility and stability of the formulation; this dose had also been shown to be effective against infection with the yellow fever and West Nile viruses in small models (25, 26). The *in vitro* activity of T-705 against these flaviviruses is comparable to the activity of the compound against the murine norovirus (21, 27). In recent study, 8 weeks of treatment with 600 mg/kg/day of T-705, was reported to result in undetectable levels of MNV-3 RNA in stool of some of the infected mice (28). At day 55 pi (i.e. about 4 weeks after stop the first round of treatment), both earlier treated groups were again treated with either compound for 14 consecutive days (Fig. 3B). Also the second treatment round with T-705 did not result in a reduction of viral shedding (Fig. 3B). The second round of 14-day treatment with 2CMC proved again effective in reducing the shedding of virus in the stool to undetectable levels (Fig. 3B), but following stop of treatment, viral rebound was observed. Despite the fact that after the first 14-day 2CMC-treatment period viral rebound had been observed, the infection proved still susceptible to the drug, suggesting that no

drug-resistant variants had emerged. We tried but failed to sequence viral RNA isolated from stool samples of days 69 and 71 pi (days which followed the end of treatment).

Although 2CMC is a relatively weak *in vitro* inhibitor of MNV replication, the compound effectively reduces virus shedding in the feces of mice chronically infected with MNV. Also, no drug-resistance developed. Favipiravir, despite inhibiting *in vitro* MNV replication, was not effective in the mouse model. The activity of 2CMC in this chronic mouse infection model suggests that a (more) potent inhibitor of norovirus replication would be able to at least reduce viral replication in patients with chronic norovirus infections. In such patients, it will be very important that drug-resistant variants do not develop. Hence, anti-norovirus drugs should (like 2CMC) not readily select for drug-resistant variants; alternatively a combination of drugs may also prevent the development of resistance.

In conclusion, we demonstrated for the first time that an inhibitor of norovirus replication is able to reduce viral shedding in chronically infected animals to undetectable levels. This validates this model for the evaluation of the potential *in vivo* efficacy of novel norovirus inhibitors.

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Fig 1 (A) Weight variation ± SEM and (B) viral RNA loads in stool samples of MNV.CR6-infected AG129 mice. Data in panel B are presented as log_{10} RNA copy numbers per gram of stool for each group. The dotted line represents the limit of detection.

Fig 2 (A) Scheme of CR6 infection and treatment with 2CMC 100 mg/kg/day for 5, 7 or 11 days, starting on day 7 pi; (B) Viral RNA loads in stool samples of MNV.CR6-infected AG129 mice untreated (n=9) or treated with 2CMC 100 mg/kg/day, starting on day 7 pi for a period of respectively 5 (n=4), 7 (n=4) or 11 (n=4) days. Data are only presented for mice that receive treatment; values in brackets represent number of treated mice for a particular time point (C-E) Follow-up period after the end of (C) 5, (D) 7 or (E) 11 days of treatment. Data are presented as log_{10} RNA copy numbers per gram of stool for each group. The dotted line represents the limit of detection. P values were determined with the nonparametric Mann-Whitney test, where *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, and ns is $p \geq 0.05$.

Fig 3 Viral RNA loads in stool samples of MNV.CR6-infected AG129 mice untreated (n=5, open squares), treated with 2CMC 100 mg/kg/day (n=10, black circles), or T-705 200 mg/kg/day (n=5, black triangles), starting on day 7 pi for a (A) first or (B) second period of 14 consecutive days (with a 4 week drug-free interval). Data are presented as log_{10} RNA copy numbers per gram of stool for each group. The dotted line represents the limit of detection. P values were determined with the nonparametric Mann-Whitney test, where *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, and ns is $p \geq 0.05$.